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DETECTION OF NOVEL PAPILLOMAVIRUS-LIKE SEQUENCES IN PARAFFINE-EMBEDDED SAMPLES OF FELINE INVASIVE AND IN SITU SQUAMOUS CELL CARCINOMA

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Meiner Familie

Il pensiero successivo è immancabilmente più saggio. (Euripide)

DETECTION OF NOVEL PAPILLOMAVIRUS-LIKE SEQUENCES IN PARAFFINE-EMBEDDED SPECIMENS OF INVASIVE AND IN SITU SQUAMOUS CELL CARCINOMA FROM CATS

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Abstract

Squamous cell carcinoma (SCC) is, behind Basal cell carcinoma, the second most common cancer of the skin in humans. Very similar skin cancers are also observed in veterinary medicine, specifically in cats. SCC has been linked to a variety of causative associations, including papillomavirus (PV) infection. Whereas close to 100 human PV types have been isolated and completely sequenced, only one single feline PV is known. However its association with feline SCC has been discussed controversially.
The purpose of the present study was to detect PV DNA by PCR in samples representing different types of feline SCC and to sequence the amplified DNA. Indeed PV DNA was detected in 18% of samples representing *invasive* SCC and in 23% of samples representing *in situ* SCC, whereas all non-tumoral control samples were negative. For the first time PV DNA had been detected in association with feline *invasive* SCC. Moreover, sequencing of the amplification products strongly suggested that a number of novel feline PVs had been detected.
The significance of the present report is based on the evidence that thus far unknown papillomaviruses can be found in association with various forms of feline SCC. In the future, isolation and further characterization of those viruses will contribute to novel insights into the pathogenesis of papilloma-associated diseases in felines.

Detection of novel papillomaviruslike sequences in paraffin-embedded specimens of invasive and in situ squamous cell carcinomas from cats

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Objective—To detect and partially characterize papillomavirus (PV) DNA in squamous cell carcinoma (SCC) tumor specimens from cats.

Sample Population—54 formalin-fixed paraffin-embedded skin biopsy specimens were examined. Specimens originated from Bowenoid in situ SCC (BISC; n = 21), invasive SCC (22), and skin affected by miscellaneous nonneoplastic conditions (11).

Procedures—Samples from each tissue block underwent DNA extraction after deparaffinization, and PCR assays were performed. Two sets of primers derived from PV *E1* were used. The first set of primers was designed for the narrow-range PCR assay and was able to generate amplification products of feline PV (FePV), canine oral PV, or closely related PVs. The second set of primers was selected for the broad-range PCR assay because of its ability to amplify DNA from 64 human PVs. Sequence analysis of each amplified DNA was performed.

Results—1 of the 21 specimens of BISC was positive for PV DNA on the basis of narrow-range PCR assay results, whereas all the other specimens (BISC, invasive SCC, and controls) had negative results for PV DNA. In contrast, 5 of 21 BISC specimens and 4 of 22 invasive SCC specimens were positive for PV DNA on the basis of broad-range PCR assay results. Sequence analysis revealed that only 1 specimen was infected by a virus closely related to classic FePV. In the 8 other specimens positive for PV DNA, DNA of unknown PVs was uncovered.

Conclusions and Clinical Relevance—Bowenoid in situ SCC and invasive SCC of cats may be associated with PVs of genetic diversity. (*Am J Vet Res* 2006;67:2036–2041)

Abbreviations

AK	Actinic keratosis
SCC	Squamous cell carcinoma
PV	Papillomavirus
HuPV	Human PV
FePV	Feline PV
BISC	Bowenoid in situ SCC
CaPV	Canine PV
BoPV	Bovine PV

Squamous cell carcinoma is, after basal cell carcinoma, the second most common cancer of the skin in humans.[1,2] Squamous cell carcinoma involves cancerous changes to the cells of the middle portion of the epidermal skin layer. This cancer may begin in normal skin; in skin at the site of a burn, injury, or scar; or at a site of chronic inflammation.[1] Most often, it originates from AK, a precancerous skin growth associated with sun exposure.[3] However, AK is often regarded as a form of SCC, which is confined to the epidermis; thus, AK is also referred to as in situ SCC.[3-6] A second form of in situ SCC, precancerous dermatitis (termed Bowen's disease), presents as 1 or more flat red scaly patches up to several centimeters wide, often found in large numbers.[1,4,6] In situ SCC can persist as such; regress; or develop into a third, even more malignant form, invasive SCC. Similar skin cancers are also observed in veterinary medicine, specifically in cats.[7] Squamous cell carcinoma has been linked to a variety of causative associations, which include exposure to UV or ionizing radiation; arsenic ingestion; toxic exposure to tars and oils; immunosuppression from drugs such as corticosteroids, azathioprine, and cyclosporine; and last but not least, to PV infection.[1,8-10]

Papillomaviruses are host-specific epitheliotropic DNA viruses that infect skin and mucous membranes. In general, PV infections are benign, result in a latent infection, or induce microlesions or benign neoplasias.[11-14] However, a subset of HuPVs and other animal PVs is clearly implicated in the development of cancer.[10, 14-17] Human PVs that cause mucosal and skin carcinomas in humans are referred to as high-risk PVs or epidermodysplasia verruciformis–associated HuPV types, respectively.[14]

Close to 100 HuPV types have been described on the basis of isolation of complete genomes.[13] Knowledge on the combination of biological properties and sequence similarities led to the definition of new criteria to define genera, species, types, subtypes, and variants within the Papillomaviridae family.[13]

In contrast to the numerous HuPVs, only a single FePV has been identified.[18] However, the existence of a few other FePVs has been suggested on the basis of findings from several clinical and immunohistochemical studies.[19-22]

Until now, little has been known about the presence of PVs in SCCs of cats. Investigators in 1 study[23]

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failed to uncover PV antigen in SCCs of cats, whereas findings in another study[24] revealed the presence of PV antigens in 44% of tumor specimens of BISC from cats. Furthermore, PV DNA has been uncovered in tumor specimens from fibropapillomas, another type of cutaneous proliferative disease, of cats.[25]

Similar to the human disease types, 3 varieties of SCC have been described for cats, which are AK, BISC, and invasive SCC.[26] Actinic keratosis usually occurs as a solitary lesion on sun-exposed, lightly haired areas, such as ear tips, external nares, or eyelids. White cats are predisposed for the development of such lesions. On the other hand, BISC is characterized usually by multiple well-circumscribed, hyperpigmented lesions that occur frequently on the face, neck, and limbs.[27,28] To our knowledge, comparative studies of these 2 early forms of cancer have not been performed in cats.

The purpose of the study reported here was to detect PV DNA in specimens representing the various types of SCC in cats and in specimens from feline skin with various nontumor conditions. We wanted to test whether tumor specimens from cats with SCC were more often infected by PVs than nontumor skin specimens. Two types of PCR assays, narrow and broad range, were applied to extend the range of targeted PVs as far as possible.

Materials and Methods

Tissue specimens—Tissues were obtained from the collections of the Prairie Diagnostics Services, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada; the Institut de Pathologie et de génétique, Loverval, Belgium; Rest associates, London; and, the Pathology Institute, Vetsuisse Faculty, University of Berne, Berne, Switzerland. Fifty-four formalin-fixed paraffin-embedded skin biopsy specimens were examined. Specimens originated from BISC (n = 21), invasive SCC (22), and miscellaneous skin conditions other than skin cancers (eg, allergic dermatitis; 11) that were used as negative controls. Specimens from white cats or from locations typical for AK such as ear tips and eyelids were excluded from this study. Thirty-micrometer-thick sections were cut from each tissue block, with a new disposable microtome blade for each block, before DNA extraction. Two canine warts, which had histopathologic characteristics of typical PV-induced inclusion bodies, and 1 bovine fibropapilloma served as positive controls.

DNA extraction—The protocol of Albini et al[29] was used for DNA extraction. Briefly, each section was deparaffinized twice with 1.2 mL of xylene at room temperature (approx 20°C) for 10 minutes, washed with 100% ethanol, and then dried at 37°C for 30 minutes. Desiccated samples were suspended in a tissue lysis buffer (50mM Tris-HCl [pH 8.5], 1mM ethilenediaminetetraacetic acid, and 2.8% sodium dodecylsulfate) and proteinase K (20 mg/mL) and incubated at 56°C on a rocking platform overnight. After lysis, samples were transferred to a column[a] and centrifuged to reduce viscosity. The DNA was precipitated with absolute ethanol and extracted with a commercial DNA kit.[b]

Primers—Two sets of primers were used for the PCR assay. Because the sequences encoding *E1* are highly conserved, the *E1* regions of FePV (GenBank accession No. AF480454) and CaPV (GenBank accession No. NC001619) were aligned to design a set of consensus primers (ie, PapF, 5′-ATGGCGGGMTARAAAAGGTA-3′ and PapR, 5′-AACAGCTGYTTTTTARCYTTTTT-3′) for narrow-range PCR assay, which is able to generate amplification products of approximately 341 bp of FePV, CaPV, or closely related PVs.

The second set of primers (ie, CP4, CP5, and PPF1 primers), also derived from *E1*, was selected for broad-range PCR assay with the objective of amplifying as many PVs as possible. With this set of primers, up to 64 HuPVs are identifiable.[30] The expected size of the PCR product was approximately 450 bp.

PCR assay and agarose gel electrophoresis—Polymerase chain reaction conditions for PapF and PapR were performed. Volumes of 30 mL were used. Each reaction contained 1 µL of genomic DNA, 200µM of each deoxynucleoside triphosphate, 0.3µM of each of the sense and antisense primers, and 2.5 units of a DNA polymerase.[c] After an initial denaturation step at 95°C for 4 minutes, PCR assay was performed for 30 cycles at 95°C for 1 minute, 50°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 74°C for 5 minutes. Deoxyribonucleic acid extracted from canine warts served as positive control, whereas DNA- and RNA-free water was used as negative control.

The PCR mix with CP4, CP5, and PPF1 primers was identical to the mix for narrow-range PCR assay, except that 0.45µM of the CP4 and CP5 primers and 0.3µM of the PPF1 primer were used. The PCR assay consisted of a denaturation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 1 minute, 47°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 75°C for 5 minutes. An extract from 1 bovine fibropapilloma served as an additional positive control.

Polymerase chain reaction products were segregated by agarose gel electrophoresis, and bands were viewed under UV light after ethidium bromide staining. Bands on the gel were excised, and DNA was extracted with a gel extraction kit.[d] Amplified DNA was sequenced by use of fluorescent sequencing and terminator chemistry.[e]

Sequence analysis—Samples were considered positive for PV DNA if they met the following requirements: they had a band of the expected size after gel electrophoresis and the sequenced DNA had homology with *E1* of previously sequenced PVs. Homologous DNA sequences were searched for by use of the National Center for Biotechnology Information GenBank via a BLAST search.[f] Sequence alignments and phylogenetic trees were made from the clustal algorithm obtained by use of a software program.[g]

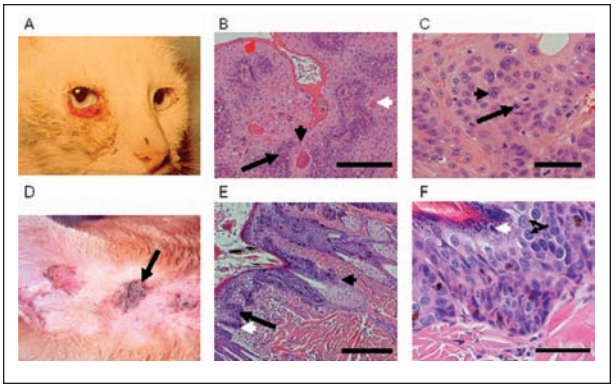
Results

Macro- and microscopic analysis—A careful macroscopic selection and microscopic confirmation of affected specimens was a major prerequisite prior to the virologic analysis. Specimens representing invasive SCC had been resected from sun-exposed or white areas of 21 domestic shorthaired cats and 1 Persian cat (12 males and 10 females). Twenty-two specimens from ear tips (n = 11), nose (3), eyelids (3), digits (3), and lips (2) met criteria required for invasive SCC. These criteria included the macroscopic presence of scaly-to-crusty and erosive-to-plaque-like or ulcerative lesions (**Figure 1**). The growth process was always endophytic. Histologically, cords or islets of infiltrative cells were detected in all specimens. Furthermore, anisocytosis; anisokaryosis; large, hyperchromatic nuclei; prominent nucleoli; increased mitotic index; and abnormal mitoses were encountered in all specimens with variable intensity and in variable proportion. In addition, keratin pearls

and intercellular bridges were present in 16 and 12 specimens, respectively.

A second group of 21 tumor specimens met the criteria for BISC. These specimens were obtained from the face (n = 16), neck (12), and limbs (3) or were scattered (2). Thirteen domestic shorthair cats, 3 domestic longhair cats, 2 Siamese, 1 Persian, 1 Himalayan, and 1 Cornish Rex were affected. Macroscopically, the lesions were squamous crustosus and grossly circular. Two lesions had a single center, but 19 were multicentric (Figure 1). Microscopically, the following criteria were met for BISC: moderate-to-severe parakeratotic hyperkeratosis, acanthosis with papillomatous hyperplasia (n = 1) or irregular hyperplasias (20), loss of polarity, and scattered dyskeratotic keratinocytes atypia in all layers of the epidermis and usually also in the infundibulum and reaching the isthmus. Furthermore, the following types of atypia were recorded: enlarged nuclei, anisokaryosis, monster cells (bizarre multinucleated giant cells), and abnormal mitotic figures. Hyperpigmentation was found in all but 3 specimens. Fifteen of the 21 specimens had clumped keratohyalin granules, which were considered as suggestive for PV infection. However, other signs such as koilocytosis and nuclear inclusion bodies were not detected.

With the exception of the ulcerated lesions, the dermis of all samples was considered normal and not heavily inflamed (Figure 1). Thus, a total of 22 samples representing invasive SCC and 21 samples representing BISC were available for virologic analysis by PCR assay and sequencing.



4c

Figure 1—Macroscopic and microscopic lesions of SCCs in cats. A—Photograph of invasive SCC in a cat with ulceration of the eyelid. B—Photomicrograph of a section of the invasive SCC from panel A at low magnification. Notice invasive proliferation of atypical keratinocytes with pearl formation (white arrow) and the cornified layer (red arrow). The basal membrane is not discernible. The dermis (long arrow) is invaded by cords of atypical keratinocytes (short black arrow, pointing towards such an invasive site). H&E stain; bar = 200 µm. C—Photomicrograph of a section of the invasive SCC from panel A at high magnification. Notice islets of keratinocytes with features of malignancy, such as anisokaryosis, anisocytosis, multinucleated cells (short arrow), and abnormal mitosis (long arrow). H&E stain; bar = 50 µm. D—Photograph of BISC in a cat with circular, crusted, erosive, and hyperpigmented plaques (arrow). E—Photomicrograph of a section of the BISC from panel D at low magnification. The basal membrane (white arrow) is intact, and the dermis is not invaded. Irregular acanthosis (long black arrow) is obvious. Notice that hair follicles are affected (short arrow). H&E stain; bar = 200 µm. F—Photomicrograph of a section of the BISC from panel D at high magnification. Notice acanthosis, hyperpigmentation (brown cells), clumped keratohyalin granules (white arrow), loss of polarity, and anisokaryosis (branched arrow). H&E stain; bar = 50 µm.

PCR assays—The narrow-range PCR assay amplified PV DNA extracted from canine warts but not DNA extracted from bovine fibropapilloma (**Figure 2**). In contrast, the broad-range PCR assay amplified PV DNA from canine warts and bovine fibropapilloma. It was concluded that both PCR assays were able to specifically amplify selected PV DNAs.

The narrow-range PCR assay was applied to samples from invasive SCC and BISC specimens; 1 BISC sample (BISC sample No. 15; **Appendix**; **Figure 3**) had positive results for PV DNA, whereas the others had negative results. Next, the broad-range PCR assay was applied to the same samples. Interestingly, 5 of 21 BISC samples (BISC sample Nos. 2, 5, 6, 10, and 15) as well as 4 of 22 SCC samples (SCC sample Nos. 15, 24, 28, and 29) had positive results for PV DNA (Figure 2). One of the samples that had positive results for PV DNA on the broad-range PCR assay (BISC sample No. 15) also had positive results for PV DNA on the narrow-range PCR assay. These results suggested that the broad-range PCR assay was indeed able to uncover PVs that were different from the known FePV and CaPVs.

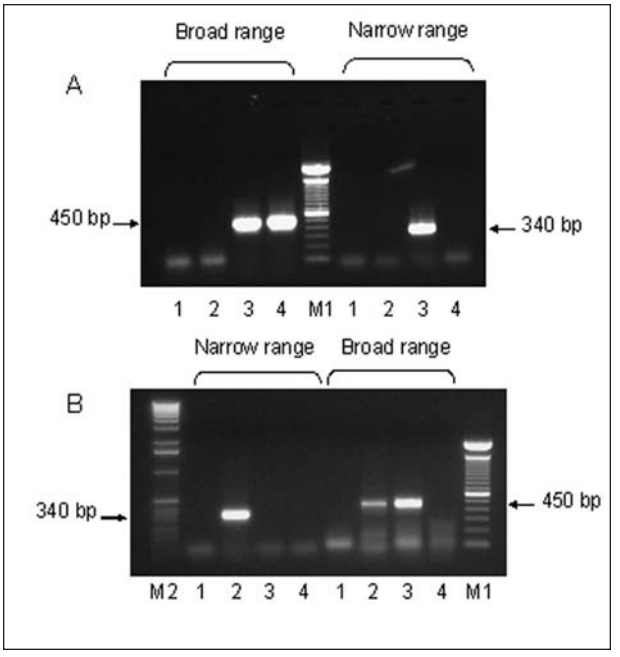


Figure 2—Establishment of broad-range and narrow-range PCR assays for detection of carnivore PVs. Polymerase chain reaction products were loaded on agarose gels and stained with ethidium bromide. A—Amplification of cloned DNA by either broad-range (450 bp) or narrow-range PCR assay (341 bp). Lane 1 = Water in place of DNA added to the reaction. Lane 2 = DNA from a commercially available phagemid.[h] Lane 3 = DNA from oral CaPV cloned into the phagemid.[h] Lane 4 = DNA from CPV3 (GenBank accession No. DQ295066) cloned into the phagemid.[h] M1 = Molecular weight marker (100-bp ladder). B—The DNA was extracted from tissues before being amplified by either the narrow range or the broad-range PCR assays. M2 = 1-kilobase ladder. M1 = 100-bp ladder. Lane 1 = Negative control with no DNA added to the reaction. Lane 2 = Extract from canine wart tissue, which had typical PV-induced inclusion bodies on histologic examination. Lane 3 = Extract from a tumor specimen of a cat with SCC (GenBank accession No. DQ085784). Lane 4 = Extract from SSC sample No. 39.

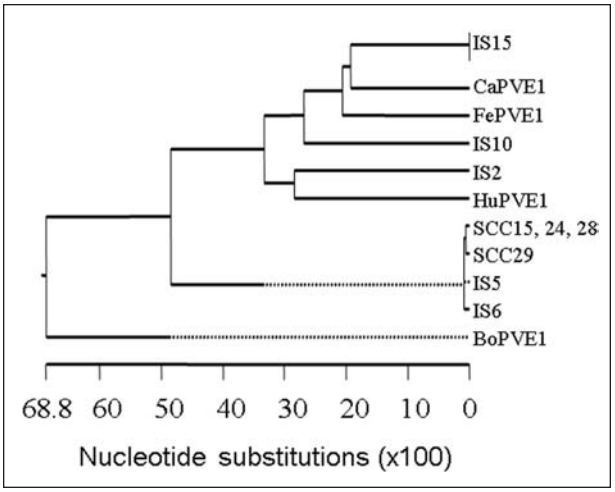


Figure 3—Phylogenetic relationships of the newly detected PV sequences with known PV *E1* sequences (ie, CaPV, FePV, HuPV, and BoPV are represented by GenBank accession Nos. D55633, AF377865, AY330623, and AJ620206, respectively). IS = BISC. SCC= Invasive SCC. Units indicate the number of substitution events (percentage of nucleotides),

Sequence analysis—The nucleotide sequence of the amplified DNA was determined and the resulting sequences were compared to assess whether novel PV-like sequences had been detected. Indeed, use of the basic local alignment search tool[e] revealed relatedness to PV *E1* sequences for all 9 samples that were positive for PV DNA. Relation to HuPV, FePV, CaPV, rat PV, and BoPV was evident. Clustal alignments[g] revealed the various degrees of relationship of the newly determined sequences among each other as well as in comparison with known PVs. Overall, CaPVs and FePVs were most closely related to the newly detected viral sequences, with a relative amino acid identity of 56% to 71%. Among the HuPVs, types 4, 55, 63, 65, 71, and 74 were aligned most frequently but type 71 most often had the closest relationship to the new sequences with 58% to 61% amino acid identity. Among the BoPVs, type 5 was the closest relative, having 55% to 62% amino acid identity. A phylogenic tree drawn from the aligned sequences divided the new sequences into 4 clusters (Figure 3). In cluster 1, BISC sample No. 15 was situated most closely with CaPV and FePV. Cluster 2 was occupied by BISC sample No. 10 and was between FePV and HuPV type 71. Cluster 3 was represented by BISC sample No. 2 and found close to HuPV type 71. The remaining sequences (SCC sample Nos. 15, 24, 28, and 29 and BISC sample Nos. 5 and 6) represented a fourth cluster, which was clearly distinct from BoPV type 5 on the most distant side and HuPV type 71, FePV, and CaPV on the less distant side. These results suggested the presence of thus far unidentified PVs in tissues representing invasive SCC and BISC. Interestingly, sequences obtained from specimens of 3 cats with invasive SCC (SCC sample Nos. 15, 24, and 28) had identical sequences. Furthermore, it was observed that not a single sample from invasive SCC specimens had been associated with the more classic FePV and CaPV. However, because the classification of PVs is based on the sequence of *L1*, the exact taxonomic position of these novel PV-like sequences could not be assigned.

Discussion

The purpose of our study was to detect and partially characterize PV DNA in samples representing in situ and invasive types of SCC in cats to learn more about PV variants in cats and about possible associations of these viruses with individual forms of SCC in cats. Two types of PCR-assays, a narrow range and a broad-range PCR, were applied to extend the range of targeted PVs as far as possible.

Careful macroscopic selection and microscopic confirmation resulted in the identification of 22 samples representing invasive SCC and 21 samples representing BISC, which were available for virologic analysis by PCR assay. Papillomavirus DNA was detected in 4 of 22 samples representing invasive SCC and in 5 of 21 samples representing BISC, whereas all nontumor control samples had negative results for PV DNA. Only 1 (BISC sample No. 15) of the 9 viral DNAs had been revealed by the narrow-range PCR assay. However, the same narrow-range PCR assay amplified PV DNA extracted from canine warts, which was expected because the primers had been chosen for their homology with conserved sequences within *E1* of FePV and CaPV. Yet, the restricted range of these primers was confirmed, as they proved unable to amplify DNA from the more distantly related bovine fibropapilloma virus. These results indicate that the remaining samples with positive results for PV DNA did not harbor conventional FePV or CaPV.

Eight samples, which had negative results for PV DNA on narrow-range PCR assay, had positive results for PV DNA on broad-range PCR assay. The broad-range PCR assay made use of a second set of primers that were also derived from *E1* but known to uncover a large variety of HuPVs.[30] In our study, this second set of primers also amplified DNA from bovine fibropapilloma virus as well as viral DNA from canine warts. Sequencing of the amplification products obtained from the 8 samples revealed novel PV-related DNAs, although relations to HuPV, FePV, CaPV, rat PV, and BoPV were evident. A phylogenic tree drawn from the aligned sequences divided the new sequences into 4 clusters. Three of those clusters had close relationship to CaPV, FePV, and HuPV. Interestingly, the fourth cluster, represented by 6 amplification products, was clearly distinct from BoPV type 5 and from HuPV type 71, FePV, and CaPV. Judging from the limited sequence information available, it appeared as if this fourth cluster represented a novel group of FePVs that had not been detected previously and that may be associated with SCC in cats. Notably, all PVs detected in association with invasive SCC were found to belong to this novel cluster.

This represents, to our knowledge, the first evidence of thus far unknown PV-like sequences associated with SCC in cats. Interestingly, some of the novel sequences were found in association with invasive SCC. Notably, previous attempts to detect conventional PV antigens in such lesions had failed,[23] which led to the hypothesis that invasive carcinomas of cats are probably not virally induced, whereas instances of Bowen's disease in cats are probably PV-induced.[7] Our findings clearly challenge the former opinion, although

a causative correlation between the disease and the novel PV strains has not yet been shown. Results of another study[24] did reveal PV antigens in 44% of BISCs. Although the proportion of BISC samples with positive results for PV DNA in our study is lower (5 of 21 BISC samples), it should be kept in mind that the broad-range PCR assay may not be able to reveal all variants of FePVs. Furthermore, it is well-known that PCR detection of viral nucleic acids in formalin-fixed and paraffin-embedded tissues may be decreased in comparison to fresh tissue.[29] Finally, the absence of PV DNA in SCC samples can also be explained by the so-called hit-and-run model, which postulates an initial transformation of the infected cell and a subsequent loss of PV DNA.[31]

Full proof of the existence of the novel PVs that are predicted through the results of our study still needs to be provided. However, we suggest that a great diversity of FePVs may exist that is in need of detection and characterization. The future use of the technique applied here will help in identifying more affected cats with papilloma-associated diseases. Virologic studies can be initiated with the aim to better characterize these novel viruses. Cloning and sequencing of the entire genomes of these viruses will allow phylogenetic comparisons with HuPVs as well as discrimination between benign and high-risk variants. Such studies can eventually provide insights into the molecular pathways underlying the pathogenesis of these viruses in cats.

a. QIAshredderTM column b, Qiagen, Basel, Switzerland.
b. QIAamp DNA Mini Kit, Qiagen, Basel, Switzerland.
c. Pfu Turbo DNA polymerase, Stratagene, La Jolla, Calif.
d. QIAquick gel extraction kit, Qiagen, Basel, Switzerland.
e. AB-3100-based fluorescent sequencing and BigDye terminator chemistry, Synergene Biotech GmbH, Biotech Center Zurich, Schlieren, Switzerland.
f. NCBI BLAST, National Center for Biotechnology Information, Bethesda, Md. Available at: www.ncbi.nlm.nih.gov/BLAST/. Accessed Oct 10, 2005.
g. Lasergene Biocomputing Software for the Macintosh, version x.x, DNAStar Inc, Madison, Wis.
h. pBluescript, Stratagene, La Jolla, Calif.

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Appendix

New papillomaviruslike sequences.

GenBank accession No.	Sample type and No.	Sequences
DQ085782	BISC 15	1 ATGGTACAAT GGGCATTTGA CAATAAGTAC ACAGATGAAG CAGAGATAGC TTTTCATTAT 61 GCACGTTTGG CAGAGGAGGA TGCAAATGCA GAGGCTTGGT TAAAAAGCAA CTCCCAAGCT 121 AAATATGTCC GAGATTGTGC GCAAATGGTG AAGCTGTATC TTAGACAAGA AATGAGGCAG 181 ACTACTATTT CTGAATGGAT TGACAAGTGC TGCCAGTCAG TGACAGAGGA CGGTGACTGG 241 GGGGATATTA TGCGCTTCTT AAAATATCAG CAAGTTAATT TCACTCAGTT TTTAACTGCC 301 ATGAGAAATG CTTTAGAGGG TAAACCTAAA AAAAACTGCT TAGTATTTTA TGGGCCTCCA 361 GATACTGGCA AGTCATATTT CTGCTTTAGT TTGGTTAGTT TTATGCAGGG GAAAGTGGTG 421 AATTTTATGA ATAGCAA
DQ085783	BISC 2	1 ANGAGGAACG ATATAGCCTA CCACTATGCA TTGCTAGCCG ACGAGGACAC AAATGCAGCG 61 GCATGGCTAG GTACAAACTC ACAGGCCAAG CATGTCAGGG ACTGCGCAGT GATGGTCAAG 121 CATTACAGGC GTGCCATAAT GTCTGCCATG AGTATGTCCG AATGGATAAA CAGACGAATG 181 GGCCTGATAG AGGAGGAAGG AGACTGGAAA AACATAGGCA ATTTCCTCAG ATACCAGGGT 241 ATAGAGGTTA TTACATTTAT AGGGGCGCTG AGGGACATGT TAAAGGGCAT TCCAAAAAGG 301 ACATGTATGT GTATAGTGGG ACCACCAGAC ACAGGGAAAT CAGCGTTTTG CCTTAGCCTG 361 CTAGACTTCT TCGGGGGTAG GGTACTGTCA TTCACCAATT ACAAAAGCCA TTTTTGNTGN 421 CCNACCCTCA A
DQ085784	SCC 15, 24, and 28	1 TTATGGTACA NGTGGGCATT TGACAATAAG TACACAGATG AAGCAGAGAT AGCTTTTCAT 61 TATGCACGTT TGGCAGAGGA GGATGCAAAT GCAGAGGCTT GGTTAAAAAG CAACTCCCAA 121 GCTAAATATG TCCGAGATTG TGCGCAAATG GTGAAGCTGT ATCTTAGACA AGAAATGAGG 181 CAGACTACTA TTTCTGAATG GATTGACAAG TGCTGCCAGT CAGTGACAGA GGACGGTGAC 241 TGGGGGGATA TCATGCGCTT CTTAAAATAT CAGCAAGTTA ATTTCACTCA GTTTTTAACT 301 GCCATGAGAA ATGCTTTAGA GGGTAAACCT AAAAAAAACT GCTTAGTATT TTATGGGCCT 361 CCAGATACTG GCAAGTCATA TTTCTGCTTT AGTTTGGTTA GTTTATGCAT GGAAAGTGGA 421 TTTNA
DQ085785	SCC 29	1 TTATGCACGT TTGGCAGAGG AGGATGCAAA TGCAGAGGCT TGGTTAAAAA GCAACTCCCA 61 AGCTAAATAT GTCCGAGATT GTGCGCAAAT GGTGAAGCTG TATCTTAGAC AAGAAATGAG 121 GCAGACTACT ATTTCTGAAT GGATTGACAA GTGCTGCCAG TCAGTGACAG AGGACGGTGA 181 CTGGGGGGAT ATTATGCGCT TCTTAAAATA TCAGCAAGTT AATTTCACTC AGTTTTTAAC 241 TGCCATGAGA AATGCTTTAG AGGGTAAACC TAAAAAAAAC TGCTTAGTAT TTTATGGGCC 301 TCCAGATACT GGCAAGTCAT ATTTCTGCTT TAGTTTGGTT AGTTTATGCT TGAAAGTGGA
DQ085786	BISC 6	1 TTTTATGGTA CAGTGGGCAT TTGACAATGA ATACTTTGAG GAAAGTGAGA TAGCATATCA 61 GTATGCATGC CTTGCAGAAA CAGAAGAAAA TGCTGCAGCC TTCCTAAATT CTAACAGCCA 121 AGCTAAGCAT GTCAGGGACT GTGCAACTAT GTGCAGATAT TATAAGAGAG CAGAAATGCA 181 GAGAATGTCA ATGTCCGCCT GGATTCACAA GAGATGTAAG GAGACCAGCC TGCAGGGAGA 241 TTGGAAAGAA ATAGTCAAGT TTCTTAGACA TCAAAGTGTA GAGTTTATTA CCTTTCTCTG 301 CAGCTTCAAG AAATTTCTCA GGGGTGTGCC TAAAAAAAAT TGCATGCTTT TCTGGGGTCC 361 TCCTAACACA GGCAAATCTA TGTTTTGCAT GAGCTTACTT TCTTTCCTAA AGGCANAGAT 421 TCTTTANC
DQ085788	BISC 5	1 TTCTTATGGT ACAGTGGGCA TTTGACAATA AGTACACAGA TGAAGCAGAG ATAGCTTTTC 61 ATTATGCACG TTTGGCAGAG GAGGATGCAA ATGCAGAGGC TTGGTTAAAA AGCAACTCCC 121 AAGCTAAATA TGTCCGAGAT TGTGCGCAAA TGGTGAAGCT GTATCTTAGA CAAGAAATGA 181 GGCAGACTAC TATTTCTGAA TGGATTGACA AGTGCTGCCA GTCAGTGACA GAGGACGGTG 241 ACTGGGGGGA TATTATGCGC TTCTTAAAAT ATCAGCAAGT TAATTTCACT CAGTTTTTAA 301 CTGCCATGAG AAATGCTTTA GAGGGTAAAC CTAAAAAAAA CTGCTTAGTA TTTTATGGGC 361 CTCCAGATAC TGGCAAGTCA TATTTCTGCT TTAGTTTGGT TAGTTTATGC AGGGAAAGTG 421 TATTTAAAA
DQ085789	BISC 10	1 TTTTTNTGGT NNCCAGTGGC NTACGATAAC GACTTCCGTG ACGAGTGCCA AATTGCCTAC 61 GAATATGCAC GGCTTGCCAC GGAGGACAGC AATGCATTGG CATGGTTGGA ATGCAATAAT 121 CAGGCCAAAT TTGTCAAAGA CTGTGCACGT ATGGTCGGGT ACTATAAGCG CGCTGAAATG 181 CAAAATATGT CTATCTCTGC TTGGATACNT AAGCAAATTA AAGATAGGCA GTGCACTACC 241 GATTGGAAAG TAATTNTGAA TTTTCNTAAG TTTCANCATG TGGAGGTTAT AATTTTTTTA 301 AATGCAATGA TGCATTTGCT CCGTGGCACG CCAAAGAAAA ATTGTCTGGT TCTGTACGGT 361 CCCCCAAATA CAGGGAAATC CATGTTCGCA ATGAGCTTAA TTCAGTGTCT GAAAGGACGT 421 GTATTGTNGT ATGTGAATTC ACGTAGTCAG TTNTGGTTGC ANCCCTTGGC AGATGCAAAA 481 ATAGCACTGC TGGACGATGC AACCAGACCA TGCTGGGAAC TATATAGATA TTTATTGAGA 541 AATGCATTGG ATGGTAATCC TATATGCCTG ACTAANCNAG C

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